invitrogen™

# Voyager™ Protein Production Kits

## Rapid cloning and expression of VP22 fusion proteins in *E. coli* for translocation of purified protein into mammalian cells

**Catalog nos. K4860-01, K4860-02**

**Version F**
13 May 2010
*25-0354*

User Manual

# Table of Contents


Important Information ..... v
Accessory Products ..... viii

**Introduction ..... 1**

Overview ..... 1

**Methods ..... 7**

PCR Primer Design ..... 7
Producing PCR Products ..... 10
TOPO® Cloning and Transformation ..... 11
Optimizing the TOPO® Cloning Reaction ..... 16
Expression of the PCR Product ..... 17
Purification of VP22 Fusion Proteins ..... 23
Application and Detection of Purified Protein in Mammalian Cells ..... 29

**Appendix ..... 32**

pCR®T7/VP22 TOPO TA Cloning® Control Reactions ..... 32
Purifying PCR Products ..... 35
Addition of 3´ A-Overhangs Post-Amplification ..... 37
pCR®T7/VP22-1-TOPO® ..... 38
Map of pCR®T7/VP22-1 ..... 42
Map of pCR®T7/VP22-2 ..... 43
Recipes ..... 44
Technical Service ..... 48
Purchaser Notification ..... 49
Product Specifications ..... 52
References ..... 53

# Important Information

## Kits

This manual is supplied with the following products.

| Kit | Catalog no. |
|---|---|
| Voyager Protein Production Kit 1 with pCR®T7/VP22-1-TOPO® | K4860-01 |
| Voyager Protein Production Kit 2 with pCR®T7/VP22-2-TOPO® | K4860-02 |

## Shipping/Storage

Each Voyager™ Protein Production Kit is shipped on dry ice. Each kit contains three boxes as listed below. Upon receipt, store each box as described in the table below.

| Box | Contents | Storage Temp |
|---|---|---|
| 1 | pCR®T7/VP22-1 TOPO TA Cloning® Reagents **OR** pCR®T7/VP22-2 TOPO TA Cloning® Reagents | -20°C |
| 2 | One Shot® TOP10 Competent Cells | -80°C |
| 3 | One Shot® BL21(DE3)pLysS Competent Cells | -80°C |

## pCR®T7/VP22 TOPO TA Cloning® Reagents

The pCR®T7/VP22 TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.** Store Box 1 at -20°C.

| Item | Concentration | Amount |
|---|---|---|
| pCR®T7/VP22-1-TOPO® **OR** pCR®T7/VP22-2-TOPO® | 10 ng/µl plasmid DNA in:<br>50% glycerol<br>50 mM Tris-HCl, pH 7.5<br>1 mM EDTA<br>1 mM DTT<br>0.1% Triton X-100<br>100 µg/ml BSA<br>30 µM phenol red | 20 µl |
| 10X PCR Buffer | 100 mM Tris-HCl, pH 8.3 (at 42°C)<br>500 mM KCl<br>25 mM $MgCl_2$<br>0.01% gelatin | 100 µl |
| dNTPs Mix | 12.5 mM dATP<br>12.5 mM dCTP<br>12.5 mM dGTP<br>12.5 mM dTTP<br>neutralized to pH 8.0 in water | 10 µl |

*continued on next page*

## pCR®T7/VP22 TOPO TA Cloning® Reagents, continued

| Item | Concentration | Amount |
|---|---|---|
| Salt Solution | 1.2 M NaCl<br>0.06 M $MgCl_2$ | 50 µl |
| Sterile Water | -- | 1 ml |
| VP22 Forward Sequencing Primer<br>(supplied with the Voyager™ Protein Production Kit 1 only) | Lyophilized in TE, pH 8 | 2 µg |
| *myc*-His Reverse Sequencing Primer<br>(supplied with the Voyager™ Protein Production Kit 1 only) | Lyophilized in TE, pH 8 | 2 µg |
| T7 Promoter Sequencing Primer<br>(supplied with the Voyager™ Protein Production Kit 2 only) | Lyophilized in TE, pH 8 | 2 µg |
| VP22 Reverse 2 Sequencing Primer<br>(supplied with the Voyager™ Protein Production Kit 2 only) | Lyophilized in TE, pH 8 | 2 µg |
| Control PCR Template | 0.05 µg/µl in TE Buffer, pH 8 | 10 µl |
| Control PCR Primers | 0.1 µg/µl **each** in TE Buffer, pH 8 | 10 µl |
| Expression Control Plasmid<br>(pCR®T7/VP22-1 **OR** pCR®T7/VP22-2) | 0.5 µg/µl in TE Buffer, pH 8 | 10 µl |
| IPTG | 1 M | 1 ml |

## One Shot® TOP10 Reagents

The table below describes the items included in the One Shot® TOP10 chemically competent cell kit. Store at -80°C.

| Item | Composition | Amount |
|---|---|---|
| SOC Medium<br>(may be stored at +4°C or room temperature) | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 ml |
| TOP10 cells | -- | 21 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 50 µl |

*continued on next page*

# Important Information, continued

## One Shot® BL21(DE3)pLysS Reagents

The table below describes the items included in the One Shot® BL21(DE3)pLysS chemically competent cell kit. Store at -80°C.

| Item | Composition | Amount |
|---|---|---|
| SOC Medium<br>(may be stored at +4°C or room temperature) | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 ml |
| BL21(DE3)pLysS cells | -- | 21 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 50 µl |

## Sequencing Primers

The table below lists the sequence and pmoles for the primers included in the Voyager™ Protein Production Kits. If you wish to order additional T7 Promoter, see **Additional Reagents** on the next page. For the other primers, see our Custom Primer ordering service at www.invitrogen.com.

| Primer | Sequence | Amount |
|---|---|---|
| VP22 Forward | 5′-GGCCACGGCGACTCGA-3′ | 410 pmoles |
| *myc*-His Reverse | 5′-ATGACCGGTATGCATATTCAG-3′ | 311 pmoles |
| T7 Promoter | 5′-TAATACGACTCACTATAGGG-3′ | 327 pmoles |
| VP22 Reverse 2 | 5′-GGTGCTAAAGTGCAGC-3′ | 406 pmoles |

## Genotype of TOP10 Cells

Use this strain for general cloning. Note that this strain cannot be used for single-strand rescue of DNA.

**Genotype:** $F^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L ($Str^R$) *end*A1 *nup*G

## Genotype of BL21(DE3)pLysS Cells

Use these cells for expression purposes **only**. Do not use these cells for propagating or maintaining your construct. For more information about the BL21(DE3)pLysS strain, see page 4.

**Genotype:** $F^-$ *ompT* *hsd*$S_B$ ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS ($Cam^R$)

The DE3 designation means this strain contains the lambda DE3 lysogen that carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase.

The pLysS plasmid ($Cam^R$) carried by the BL21(DE3)pLysS strain produces T7 lysozyme to reduce basal level expression of the gene of interest. pLysS confers resistance to chloramphenicol and contains the replication origin from plasmid p15A. This origin allows pLysS to be compatible with plasmids containing origins derived from pUC or pBR322. For more information on pLysS, see page 5.

# Accessory Products

## Additional Reagents

The products listed in this section are intended for use with the Voyager™ Protein Production Kits. For more information, visit www.invitrogen.com or contact Technical Service (see page 48).Ordering information for these reagents is provided below.

| Item | Amount | Catalog no. |
|---|---|---|
| *Taq* DNA Polymerase, Native | 100 units | 18038-018 |
| | 500 units | 18038-042 |
| *Taq* DNA Polymerase, Recombinant | 100 units | 10342-053 |
| | 500 units | 10342-020 |
| Platinum® *Taq* DNA Polymerase High Fidelity | 100 units | 11304-011 |
| One Shot® TOP10 Chemically Competent *E.coli* | 10 reactions | C4040-10 |
| | 20 reactions | C4040-03 |
| | 40 reactions | C4040-06 |
| One Shot® TOP10 Electrocompetent *E. coli* | 10 reactions | C4040-50 |
| | 20 reactions | C4040-52 |
| One Shot® BL21(DE3)pLysS Chemically Competent *E. coli* | 10 reactions | C6060-10 |
| | 20 reactions | C6060-03 |
| One Shot® BL21(DE3)pLysE Chemically Competent *E. coli* | 20 reactions | C6565-03 |
| One Shot® BL21(DE3) Chemically Competent *E. coli* | 20 reactions | C6000-03 |
| T7 Promoter Sequencing Primer | 2 µg (327 pmoles) | N560-02 |
| Kanamycin | 5 g | Q100-18 |
| imMedia™ Kan Liquid | 20 pouches | Q610-20 |
| imMedia™ Kan Agar | 20 pouches | Q612-20 |

*continued on next page*

# Accessory Products, continued

## Detection of Fusion Protein

A number of antibodies are available from Invitrogen to detect expression of your VP22 fusion protein from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection in western blots using colorimetric or chemiluminescent detection methods. FITC-conjugated antibodies allow one-step immunofluorescence detection of VP22 fusion proteins. The amount of antibody supplied is sufficient for 25 westerns at a 10 ml working solution (primary and HRP-conjugated antibodies only) or for 25 immunostaining reactions at a 1 ml working solution (FITC-conjugated antibodies only).

| Antibody | Epitope | Catalog no. |
|---|---|---|
| Anti-*myc* | Detects the 10 amino acid epitope derived from *c-myc* (Evans *et al.*, 1985): EQKLISEEDL | R950-25 |
| Anti-*myc*-HRP | | R951-25 |
| Anti-*myc*-FITC | | R953-25 |
| Anti-His(C-term) | Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH | R930-25 |
| Anti-His(C-term)-HRP | | R931-25 |
| Anti-His(C-term)-FITC | | R933-25 |

## Purification of Fusion Protein

The polyhistidine (6xHis) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™. Ordering information for ProBond™ resin is provided below.

| Item | Quantity | Catalog no. |
|---|---|---|
| ProBond™ Purification System | 12 ml precharged ProBond™ resin, six columns, and buffers for native and denaturing purification | K850-01 |
| ProBond™ Purification System with Anti-*myc*-HRP Antibody | 1 Kit (same components as above plus 50 µl of antibody) The amount of antibody supplied is sufficient for 25 westerns. | K852-01 |
| ProBond™ Purification System with Anti-His(C-term)-HRP Antibody | 1 Kit (same components as above plus 50 µl of antibody) The amount of antibody supplied is sufficient for 25 westerns | K853-01 |
| ProBond™ Resin | 50 ml | R801-01 |
| | 150 ml | R801-15 |
| Purification Columns | 50 polypropylene columns | R640-50 |

*continued on next page*

# Accessory Products, continued

## Other Voyager™ Protein Production Kits

Additional Voyager™ Protein Production Kits are available from Invitrogen which allow production of N-terminal or C-terminal VP22 fusion proteins containing a nuclear export signal. Once purified from *E. coli*, the VP22 fusion protein can be applied directly to mammalian cells where it is targeted to the cytoplasm. For more information, visit www.invitrogen.com or call Technical Service (see page 48).

| Item | Amount | Catalog no. |
|---|---|---|
| Voyager™ NES Protein Production Kit 1 with pCR®T7/VP22/NES-1-TOPO® | 20 reactions | K4880-01 |
| Voyager™ NES Protein Production Kit 2 with pCR®T7/VP22/NES-2-TOPO® | 20 reactions | K4880-02 |

## Other Voyager™ Kits for Expression in Mammalian Cells

Additional Voyager™ products are available from Invitrogen which allow cloning of N-terminal or C-terminal VP22 fusion proteins for transfection and direct expression of the gene of interest in mammalian cells. Once expressed, the VP22 fusion protein translocates into surrounding non-transfected cells. The Voyager™ kits listed below also facilitate creation of stable cell lines expressing VP22 fusion proteins. For more information, visit www.invitrogen.com or contact Technical Service (see page 48).

| Item | Amount | Catalog no. |
|---|---|---|
| pVP22/*myc*-His TOPO® TA Expression Kit | 20 reactions | K4840-01 |
| pVP22/*myc*-His Kit | 20 µg | V484-01 |
| pVP22/*myc*-His2 TOPO® TA Expression Kit | 20 reactions | K4850-01 |
| pVP22/*myc*-His2 Kit | 20 µg | V485-01 |

## Protein Analysis Reagents

Ordering information for products used for protein electrophoresis and blotting, staining, and protein standards are listed below. A large variety of pre-cast gels and pre-made buffers for SDS-PAGE are also available from Invitrogen. For more information, visit www.invitrogen.com or contact Technical Service (see page 48).

| Item | Amount | Catalog no. |
|---|---|---|
| SimplyBlue™ SafeStain | 1 L | LC6060 |
| SeeBlue™ Plus2 Pre-Stained Standard | 500 µl | LC5925 |
| MagicMark™ XP Western Protein Standard | 250 µl | LC5602 |
| Mark 12™ Unstained Standard | 1 ml | LC5677 |
| Nitrocellulose/Filter Paper Sandwich, 0.45 µm | 20/pack | LC2000 |
| Invitrolon™/Filter Paper Sandwich, 0.45 µm | 20/pack | LC2005 |
| Blotting Roller | 1 | LC2100 |
| WesternBreeze® Chromogenic Kit, Anti-Mouse | 1 kit | WB7103 |
| WesternBreeze® Chromogenic Kit Anti-Rabbit | 1 kit | WB7105 |
| WesternBreeze® Chemiluminescent Kit, Anti-Mouse | 1 kit | WB7104 |
| WesternBreeze® Chemiluminescent Kit, Anti-Rabbit | 1 kit | WB7106 |

# Introduction

## Overview

### Introduction

The Voyager™ Protein Production Kits utilize the TOPO® Cloning technology to facilitate rapid cloning of *Taq* polymerase-amplified PCR products into the pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® vector. The pCR®T7/VP22-1-TOPO® and pCR®T7/VP22-2-TOPO® vectors allow cloning of PCR products to the N-terminus or C-terminus, respectively, of the herpes virus VP22 protein. Once cloned into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, the VP22 fusion protein can be expressed in and purified from *E. coli*, then applied to and imported into the mammalian cells of interest. The translocation and import properties of the VP22 protein form the basis of the Voyager™ technology (see the next page).

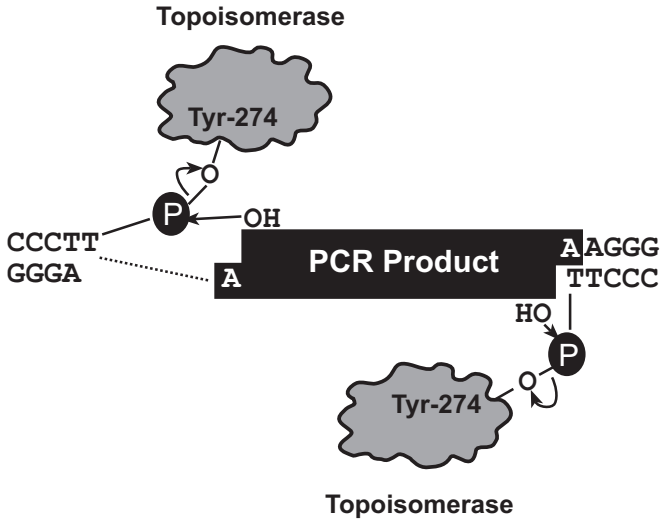
### How TOPO® Cloning Works

The plasmid vector, pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, is supplied linearized with:

- Single 3′ thymidine (T) overhangs for TA Cloning®
- Topoisomerase covalently bound to the vector (this is referred to as "activated" vector)

*Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see below).




*continued on next page*

# Overview, continued

## How TOPO® Cloning Works, continued

Once the PCR product is cloned into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® and transformants analyzed for the correct orientation, expression of the VP22 fusion protein can be induced in *E. coli* by addition of IPTG. Recombinant VP22 fusion protein is then purified using metal-chelating resin. Purified VP22 fusion protein can be applied to the mammalian cell line of choice where it is rapidly imported into virtually 100% of the cells.

## Voyager™ Technology

The Voyager™ technology utilizes the protein, VP22, one of the structural proteins that form the tegument of herpes simplex virus type 1 (HSV-1). The tegument is a region located between the capsid and the envelope. This novel protein possesses unusually potent translocation properties following transfection or infection (Elliott and O'Hare, 1997) or when simply applied to mammalian cells. The VP22 protein expressed in the Voyager™ Protein Production Kits exhibits the following properties:

- When applied to mammalian cells, the VP22 protein is rapidly imported and localized to the nucleus of the cell. Uptake occurs within 20 minutes after addition of VP22 protein to cells.
- Heterologous proteins fused to VP22 protein can be similarly imported and localized to the nucleus of the cell. This is not always the case, though, as the site of localization appears to depend in part, on the nature of the protein of interest (see page 31).
- VP22 fusion proteins can be imported into a wide variety of mammalian cell lines including those that are typically refractory to standard transfection methods.
- Heterologous proteins fused to VP22 protein appear to retain their biological activity.

For more information about the translocation properties of the VP22 protein, refer to published references (Brewis *et al.*, 2000; Elliott and O'Hare, 1997; Phelan *et al.*, 1998).

**Important**

Note that the VP22 protein expressed from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® is a truncated version of the native VP22 protein and does not contain the entire open reading frame. The VP22 protein expressed from pCR®T7/VP22-1-TOPO® and pCR®T7/VP22-2-TOPO® is derived from amino acids 159-301 of the native VP22 protein. This truncated VP22 protein appears to be sufficient to impart translocation properties to heterologous fusion partners (P. O'Hare, personal communication).

*continued on next page*

# Overview, continued

## Cell Lines Tested

The table below lists the mammalian cell lines that have been successfully tested at Invitrogen for import of VP22 fusion proteins produced using the Voyager™ Protein Production Kits. All of these are established cell lines. To date, no pleiotrophic effects have been observed in any of the uptake experiments performed using VP22 fusion proteins. Primary cell lines have not been extensively tested.

| Cell Line | Description | Adherent/Suspension |
|---|---|---|
| COS-1 | African Green Monkey kidney fibroblast (SV40 Large T antigen transformed) | Adherent |
| Swiss 3T3 | Murine embryo fibroblast | Adherent |
| CHO | Chinese hamster ovary | Adherent |
| PC12 | Rat pheochromocytoma | Suspension |
| Jurkat | Human T-cell leukemia | Suspension |

## Proteins Expressed

In experiments at Invitrogen, the following proteins have been expressed as VP22 fusions in *E. coli* using the Voyager™ Protein Production Kits. Purified VP22 fusion proteins were imported into mammalian cells and shown to be biologically active.

| Protein | Size (kDa) | Cell Line Tested |
|---|---|---|
| Human rhoA | 22 | Swiss 3T3 |
| HIV rev | 14 | CHO |
| Green Fluorescent Protein (GFP) | 27 | PC12, Jurkat, CHO |
| Bacteriophage T7 RNA polymerase | 99 | COS-1, 293 |
| β-galactosidase alpha fragment | 10 | CHO |

*continued on next page*

# Overview, continued

## The pCR®T7/VP22-1-TOPO® and pCR®T7/VP22-2-TOPO® Vectors

pCR®T7/VP22-1-TOPO® and pCR®T7/VP22-2-TOPO® are 4.9 kb expression vectors designed to facilitate rapid cloning and expression of PCR products as fusions to the VP22 protein. The vectors allow high-level, inducible expression of the gene of interest in *E. coli*. The vectors contain the following elements:

- T7 promoter for high-level, inducible expression of the gene of interest in *E. coli* (see below)
- TOPO® Cloning site for rapid cloning of *Taq*-amplified PCR products
- Truncated VP22 ORF (amino acids 159-301 only) to allow import of the gene of interest into a wide variety of mammalian cells
- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant fusion protein
- Kanamycin resistance gene for selection in *E. coli*

A control plasmid (pCR®T7/VP22-1 or pCR®T7/VP22-2) is included for use as a positive control for expression and purification (see page 17 for more information).

## Regulation of Expression of the Gene of Interest

Expression of the gene of interest from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® is controlled by the very strong bacteriophage T7 promoter that drives expression of gene 10 (ϕ10). T7 RNA polymerase specifically recognizes this promoter. For expression of the gene of interest, it is necessary to deliver T7 RNA polymerase to the cells by either inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the Voyager™ Protein Production Kit, addition of IPTG to transformed BL21(DE3)pLysS cells induces expression of T7 RNA polymerase. Once sufficient T7 RNA polymerase is produced, it binds to the T7 promoter in pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® and transcribes the gene of interest.

## Use of TOP10 Cells

One Shot® TOP10 competent cells, which do not contain T7 polymerase, are included in the Voyager™ Protein Production Kit to provide a host for stable propagation and maintenance of recombinant plasmids. The presence of T7 polymerase, even at low basal levels, can lead to expression of the desired gene even in the absence of inducer (see below). In general, this is not a problem, but if the gene of interest is toxic to the *E. coli* host, plasmid instability and/or cell death results. **We recommend that you transform your TOPO® Cloning reaction into TOP10 cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into one of the expression strains described below.

## Regulation of Expression of T7 RNA Polymerase

The BL21(DE3)pLysS *E. coli* strain is specifically included in this kit for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This lambda lysogen contains the *lacI* gene, the T7 RNA polymerase gene under the control of the *lacUV5* promoter, and a small portion of the *lacZ* gene. This *lac* construct is inserted into the *int* gene, thus inactivating the gene. Disruption of the *int* gene prevents lysis in the absence of helper phage. The lac repressor represses expression of T7 RNA polymerase. Addition of the gratuitous inducer, isopropyl β-D-thiogalactoside (IPTG) allows expression of T7 RNA polymerase.

The BL21(DE3)pLysE strain is also available from Invitrogen. For more information on BL21(DE3)pLysS and BL21(DE3)pLysE, refer to the next page.

*continued on next page*

# Overview, continued

## Regulation of T7 RNA Polymerase by T7 Lysozyme

There is always some basal level expression of T7 RNA polymerase. If a toxic gene is cloned downstream of the T7 promoter, basal expression of this gene may lead to reduced growth rates, increased cell death, or plasmid instability. T7 lysozyme has been shown to bind to T7 polymerase and inhibit transcription. This activity is exploited to reduce basal levels of T7 RNA polymerase.

**Note**

T7 lysozyme is a bifunctional enzyme. In addition to its T7 RNA polymerase binding activity, it also cleaves a specific bond in the peptidoglycan layer of the *E. coli* cell wall. This activity increases the ease of cell lysis by freeze-thaw cycles prior to purification, thus making cells more fragile.

## pLysE and pLysS

The gene for T7 lysozyme has been cloned into the *Bam*H I site of pACYC184 (Chang and Cohen, 1978; Studier *et al.*, 1990). If the gene is oriented so that it is expressed from the constitutive *tet* promoter, the plasmid is called pLysE; if it is cloned in the opposite orientation so that it is expressed from the ϕ3.8 promoter, it is called pLysS. The plasmids confer resistance to chloramphenicol (34 µg/ml) and contain the origin of replication from plasmid p15A. This origin allows pLysS and pLysE to be stably maintained with pUC- and pBR322-derived plasmids in the same host. The differences between these two plasmids are summarized in the table below.

| Feature | pLysS | pLysE |
|---|---|---|
| Relative amount of T7 lysozyme | Moderate (may not sufficiently suppress T7 RNA polymerase for expression of more toxic genes) | High |
| Growth rate of host | No or little effect | May cause a significant decrease and/or cell lysis |
| Stability of expression plasmid | Increases | Increases |
| Lag between addition of inducer and expression of desired gene | Short | Long |
| Maximum expression level of desired protein | No effect | May reduce |

Note that One Shot® BL21(DE3)pLysS competent cells are supplied in the Voyager™ Protein Production Kit. In most cases, pLysS supplies sufficient T7 lysozyme to reduce the activity of T7 RNA polymerase while maintaining good growth rates and maximum yield of recombinant protein.

If you discover that your gene is still toxic to *E. coli*, try BL21(DE3)pLysE cells (see page viii for ordering information). Contact Technical Service for more information (see page 48).

*continued on next page*

# Overview, continued

## Experimental Outline

Use the following outline to clone and express your gene of interest in pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®.

| Step | Action | Page |
|---|---|---|
| 1 | Design PCR primers to clone your gene of interest in frame with the truncated VP22 ORF and the C-terminal peptide containing the *c-myc* epitope and the polyhistidine (6xHis) tag. Consult the diagram of the TOPO® Cloning site on page 8 or page 9 to help you design your PCR primers. | 7-9 |
| 2 | Produce your PCR product. | 10 |
| 3 | TOPO® Clone your insert into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® and transform into One Shot® TOP10 *E. coli*. Select transformants on 50 μg/ml kanamycin. | 11-14 |
| 4 | Analyze your transformants for the presence and orientation of insert by restriction enzyme digestion. | 14 |
| 5 | Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the truncated VP22 ORF and the C-terminal peptide. | 14 |
| 6 | Prepare purified plasmid and transform into One Shot® BL21(DE3)pLysS *E. coli*. Select transformants on 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. | 17-18 |
| 7 | Induce expression of the VP22 fusion protein with IPTG and optimize expression conditions. | 19-20 |
| 8 | Scale-up expression and purify VP22 fusion protein from BL21(DE3)pLysS cells using metal-chelating resin such as ProBond™. | 23-28 |
| 9 | Apply purified VP22 fusion protein to mammalian cells. | 29-30 |
| 10 | Test for uptake of your recombinant VP22 fusion protein by immunofluorescence, western blot analysis, or functional assay. | 30-31 |

It is very unlikely that VP22 fusions can penetrate the skin; however, this has not been tested. Avoid contact with the skin or mucous membranes. We recommend that you wear a lab coat, gloves, and safety glasses when working with solutions containing VP22 fusions.

# Methods

## PCR Primer Design

### Introduction

The design of the PCR primers to clone your DNA sequence of interest is critical for fusion to VP22 and expression. **Remember that your PCR product will have single 3´ adenine overhangs if *Taq* is used as your polymerase (see page 37).**

**Note**

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®.

### Cloning in pCR®T7/VP22-1-TOPO®

To fuse your gene to the N-terminus of VP22, design your 5´ PCR primer such that the PCR product will clone in frame with the VP22 ORF. pCR®T7/VP22-1-TOPO® also contains a C-terminal peptide encoding the *c-myc* epitope and a polyhistidine (6xHis) tag to facilitate purification and detection of your recombinant protein. Therefore, remember to design your PCR primers such that the PCR product will also clone in frame with the C-terminal peptide.

**Note**: Cloning efficiencies may vary depending on the 5´ nucleotide sequence of your primer (see page 34).

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to page 10.

### Cloning in pCR®T7/VP22-2-TOPO®

You will need to consider the following when cloning your PCR product into pCR®T7/VP22-2-TOPO®:

- The T7 leader contains an ATG initiation codon and surrounding sequences that allow optimal expression of heterologous fusion proteins in *E. coli*. We recommend that you design your 5′ PCR primer such that your PCR product will clone in frame with the T7 leader.
- To fuse your gene to the C-terminus of VP22, design your 3′ PCR primer such that the PCR product will clone in frame with the VP22 ORF. Note that if you clone your PCR product in frame with the VP22 ORF, your PCR product will automatically be in frame with the C-terminal peptide encoding the *c-myc* epitope and a polyhistidine (6xHis) tag.

**Note**: Cloning efficiencies may vary depending on the 5´ nucleotide sequence of your primer (see page 34).

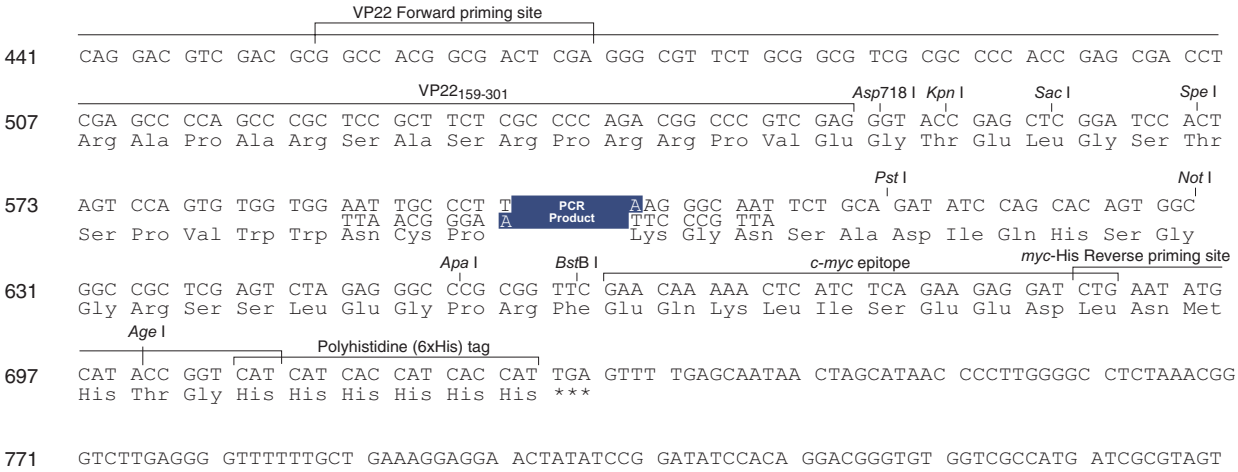
Use the diagram on page 9 to design your PCR primers. Once you have designed your PCR primers, proceed to page 10.

*continued on next page*

# PCR Primer Design, continued

## TOPO® Cloning Site of pCR®T7/VP22-1-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 597 and 598. This is the TOPO® Cloning site**. The complete sequence of pCR®T7/VP22-1-TOPO® is available for download from www.invitrogen.com or requested from Technical Service (see page 48).** For a map and a description of the features of pCR®T7/VP22-1-TOPO®, refer to pages 38-39.

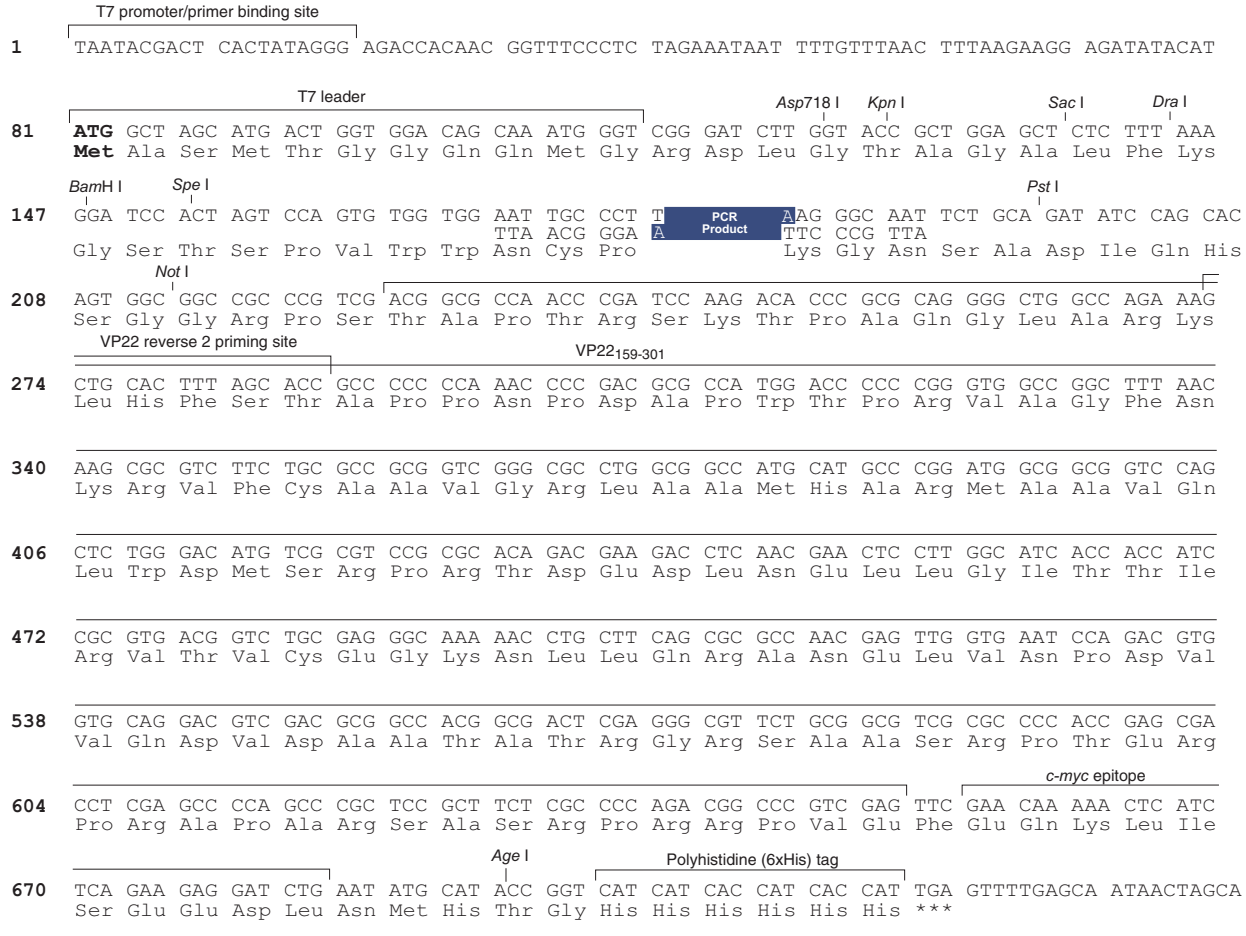

VP22 Forward priming site
441 CAG GAC GTC GAC GCG GCC ACG GCG ACT CGA GGG CGT TCT GCG GCG TCG CGC CCC ACC GAG CGA CCT
VP22159-301
Asp718 I
Kpn I
Sac I
Spe I
507 CGA GCC CCA GCC CGC TCC GCT TCT CGC CCC AGA CGG CCC GTC GAG GGT ACC GAG CTC GGA TCC ACT
Arg Ala Pro Ala Arg Ser Ala Ser Arg Pro Arg Arg Pro Val Glu Gly Thr Glu Leu Gly Ser Thr
Pst I
Not I
573 AGT CCA GTG TGG TGG AAT TGC CCT T PCR Product AAG GGC AAT TCT GCA GAT ATC CAG CAC AGT GGC
TTA ACG GGA A TTC CCG TTA
Ser Pro Val Trp Trp Asn Cys Pro Lys Gly Asn Ser Ala Asp Ile Gln His Ser Gly
Apa I
BstB I
c-myc epitope
myc-His Reverse priming site
631 GGC CGC TCG AGT CTA GAG GGC CCG CGG TTC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT ATG
Gly Arg Ser Ser Leu Glu Gly Pro Arg Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met
Age I
Polyhistidine (6xHis) tag
697 CAT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTT TGAGCAATAA CTAGCATAAC CCCTTGGGGC CTCTAAACGG
His Thr Gly His His His His His His ***
771 GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA ACTATATCCG GATATCCACA GGACGGGTGT GGTCGCCATG ATCGCGTAGT

*continued on next page*

# PCR Primer Design, continued

## TOPO® Cloning Site of pCR®T7/VP22-2-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The ATG initiation codon in the T7 leader is shown in bold. The vector is supplied linearized between base pair 180 and 181. This is the TOPO® Cloning site**. The complete sequence of pCR®T7/VP22-2-TOPO® is available for download from www.invitrogen.com or requested from Technical Service (see page 48).** For a map and a description of the features of pCR®T7/VP22-2-TOPO®, refer to pages 40-41.



# Producing PCR Products

## Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

## Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

## Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product (e.g. Expand™ or eLONGase™).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 37.

## Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using a plasmid for template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

| | |
|---|---|
| DNA Template | 10-100 ng |
| 10X PCR Buffer | 5 µl |
| 50 mM dNTPs | 0.5 µl |
| Primers (0.1-0.2 µg each) | 1 µM each |
| Sterile water | add to a final volume of 49 µl |
| *Taq* Polymerase (1 unit/µl) | 1 µl |
| Total Volume | 50 µl |

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single, discrete band, refer to the **Note** below.

**Note**

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before TOPO® Cloning into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® (see pages 35-36). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Contact Technical Service for more information (page 48).

# TOPO® Cloning and Transformation

## Introduction

TOPO® Cloning technology allows you to ligate your PCR products into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® and transform the recombinant vector into *E. coli* all in one day. It is important to have everything you need set up and ready to use to ensure you obtain the best possible results. If this is the first time you have you are performing the TOPO® reaction, we recommend performing the control reactions on pages 32-33 in parallel with your samples.

**Note**

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction results in the following:

- a 2- to 3-fold increase in the number of transformants.
- allows for longer incubation times (up to 30 minutes). Longer incubation times can result in an increase in the number of transformants obtained.

Including salt in the TOPO® Cloning reaction prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

If you do **not** include salt in the TOPO® Cloning reaction, the number of transformants obtained generally decreases as the incubation time increases beyond 5 minutes.

**Important**

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see below).** For this reason two different TOPO® Cloning reaction protocols are provided to help you obtain the best possible results. Review the following information carefully.

## Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

## Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

*continued on next page*

# TOPO® Cloning and Transformation, continued

### Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubators

### imMedia™ Agar Plates

For fast and easy microwaveable preparation of Low Salt LB media and agar containing kanamycin, imMedia™ Kan Liquid (Catalog no. Q610-20) and imMedia™ Kan Agar (Catalog no. Q611-20) are available from Invitrogen. For more information, visit www.invitrogen.com or call Technical Service (see page 48).

**Note**

**There is no blue-white screening for the presence of inserts.** Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in each kit can be used to sequence across an insert in the TOPO® Cloning site to confirm orientation and reading frame.

### Preparation for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g. add 5 µl of the Salt Solution to 15 µl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® TOP10 cells for each transformation.

**Important**

Remember to use One Shot® **TOP10** *E. coli* to transform your TOPO® Cloning reaction. The BL21(DE3)pLysS strain supplied with the kit should **only** be used for expression purposes and **not** for general cloning purposes. For more information about BL21(DE3)pLysS cells, refer to pages 4 and 17.

*continued on next page*

# TOPO® Cloning and Transformation, continued

## Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 16.

**Note**: The red or yellow color of the TOPO® vector solution is normal and is used to visualize the solution.

| Reagent* | Chemically Competent *E. coli* | Electrocompetent *E. coli* |
|---|---|---|
| Fresh PCR product | 0.5 to 4 µl | 0.5 to 4 µl |
| Salt Solution | 1 µl | -- |
| Dilute Salt Solution (1:4) | -- | 1 µl |
| Sterile Water | add to a final volume of 5 µl | add to a final volume of 5 µl |
| TOPO® vector | 1 µl | 1 µl |

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

## Performing the TOPO® Cloning Reaction

Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

**Note:** For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

Place the reaction on ice and proceed to **One Shot® Chemical Transformation** (below) or **Transformation by Electroporation** (next page). **Note:** You may store the TOPO® Cloning reaction at -20°C overnight.

## One Shot® TOP10 Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from above into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   **Note:** Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion (see above).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

*continued on next page*

# TOPO® Cloning and Transformation, continued

## Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction to 50 µl of electrocompetent *E. coli* in a microcentrifuge tube. Transfer the mixture to a 0.1 cm cuvette and mix gently. Do not mix by pipetting up and down. Avoid formation of bubbles.
2. Electroporate your samples using your own protocol and your electroporator.

   **Note:** If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for 1 hour at 37°C to allow expression of the antibiotic resistance genes.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, below).

**Note**

Addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

## Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50 µg/ml kanamycin (3-5 ml).
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01).
3. Analyze the plasmids by restriction enzyme analysis or by sequencing.
   - If you are TOPO® Cloning into pCR®T7/VP22-1-TOPO®, the VP22 Forward and *myc*-His Reverse sequencing primers are included in the kit to help you sequence your insert.
   - If you are TOPO® Cloning into pCR®T7/VP22-2-TOPO®, the T7 Promoter and VP22 Reverse 2 sequencing primers are included in the kit to help you sequence your insert.

   Refer to the diagram on page 8 or page 9 for restriction sites and sequence surrounding the TOPO® Cloning site of pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, respectively.

   **Note:** Resuspend each primer in 20 µl of sterile water to prepare a 0.1 µg/µl stock solution.

If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

*continued on next page*

# TOPO® Cloning and Transformation, continued

## Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use a combination of one of the primers included with the kit with a primer that binds within your insert. You will have to determine the amplification conditions. If this is the first time you have used this technique, we recommend that you perform restriction enzyme analysis in parallel to confirm that PCR gives you the correct result. Artifacts may be obtained because of mispriming or contaminating template.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume and multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. (Don't forget to make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using parameters previously determined (see text, above).
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

**Important**

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 32-33. These reactions will help you troubleshoot your experiment.

## Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long-term storage. We recommend that you also store the purified plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on LB plates containing 50 µg/ml kanamycin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow the culture to mid-log phase ($OD_{600}$ = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

# Optimizing the TOPO® Cloning Reaction

## Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

## Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

## More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

**Note:** Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

## Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

# Expression of the PCR Product

## Introduction

To express your VP22 fusion product from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, you will use BL21(DE3)pLysS cells included with the kit as the host strain. You will need purified plasmid DNA of your construct to transform into BL21(DE3)pLysS for expression studies. Since each recombinant protein has different characteristics that may affect optimal expression, we recommend that you perform a time course of expression to determine the optimal conditions for expression of your protein.

## BL21(DE3)pLysS

This *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter. **Each time** you wish to perform an expression experiment, you will transform your plasmid into BL21(DE3)pLysS. **Do not use this strain for propagation and maintenance of your plasmid. Use TOP10 for propagation and maintenance of your plasmid.** Basal level expression of T7 RNA polymerase may lead to plasmid instability if your gene of interest is toxic to *E. coli*. For more information on this strain, refer to page 4.

## Positive Control

The supercoiled pCR®T7/VP22-1 or pCR®T7/VP22-2 vector provided with the respective Voyager™ Protein Production Kits produce a fusion protein consisting of the T7 leader, the truncated VP22 ORF, and the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag, and may be used as a positive control for expression and purification. The table below shows the predicted size of the fusion protein that is produced from each vector. For a detailed map and a description of the features of each vector, see pages 42–43.

| Vector | Size of Fusion Protein |
|---|---|
| pCR®T7/VP22-1 | 23 kDa |
| pCR®T7/VP22-2 | 23 kDa |

To propagate and maintain the pCR®T7/VP22-1 or pCR®T7/VP22-2 vector, transform 10 ng of the plasmid into One Shot® TOP10 cells using the procedure on page 13.

## Experimental Outline

The table below outlines the basic steps needed to induce expression of your gene of interest in *E. coli*.

| Step | Action |
|---|---|
| 1 | Isolate plasmid DNA using standard procedures and transform your pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® construct and the appropriate positive control separately into One Shot® BL21(DE3)pLysS cells. Select transformants using 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. |
| 2 | Grow the transformants and induce expression with IPTG over several hours. Take several time points to determine the optimal time of expression. |
| 3 | Optimize expression to maximize the yield of protein. |

*continued on next page*

# Expression of the PCR Product, continued

## Materials to Have on Hand

Be sure to have the following solutions and equipment on hand before starting the experiment:

- 34 mg/ml chloramphenicol in ethanol (**Note:** Chloramphenicol is **required** to ensure the presence of pLysS)
- SOB or LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol
- 37°C incubator (shaking and non-shaking)
- 42°C water bath
- 1 M IPTG
- Bacterial Cell Lysis Buffer (see page 46 for recipe)
- Liquid nitrogen
- 1X and 2X SDS-PAGE sample buffer (see page 44 for a recipe for 2X SDS-PAGE sample buffer)
- Reagents and apparatus for SDS-PAGE (see page 19)
- Boiling water bath
- Sterile water

## Plasmid Preparation

Purified plasmid DNA may be isolated using your method of choice. A large variety of plasmid purification kits is available from Invitrogen. For more information, visit www.invitrogen.com.

## One Shot® BL21(DE3)pLysS Transformation Reaction

To transform your construct or the positive control (10 ng each) into One Shot® BL21(DE3)pLysS cells, follow the instructions below. You will need one vial of cells per transformation.

Note that you will not plate the transformation reaction, but inoculate it into medium for growth and subsequent expression.

1. Thaw on ice, one vial of One Shot® BL21(DE3)pLysS cells per transformation.
2. Add 5-10 ng DNA in a 1 to 5 µl volume into each vial of One Shot® BL21(DE3)pLysS cells and mix by stirring gently with the pipette tip. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 µl of room temperature SOC medium.
7. Cap the tube tightly, tape the tube on its side (for better aeration), and incubate at 37°C for 30 minutes with shaking (200 rpm).
8. Add the **entire** transformation reaction to 10 ml of LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol.
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, next page.

*continued on next page*

# Expression of the PCR Product, continued

## Pilot Expression

1. Inoculate 10 ml of LB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol with 500 µl of the overnight culture from Step 9, previous page.
2. Grow two hours at 37°C with shaking. $OD_{600}$ should be about 0.5-0.7 (mid-log).
3. Split the culture into two 5 ml cultures. Add IPTG to a final concentration of 1 mM to one of the cultures. You will now have two cultures: one induced, one uninduced.
4. Remove a 500 µl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
5. Freeze the cell pellets at -20°C. These are the zero time point samples.
6. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
7. For each time point, remove 500 µl from the induced and uninduced cultures and process as described in Steps 4 and 5. Proceed to **Preparation of Samples**, below.

## Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented NuPAGE® Gel System prevents the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits for visualization of proteins. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, visit www.invitrogen.com or call Technical Service (see page 48).

## Preparation of Samples

If you wish to analyze your samples for soluble protein, see the next section.

1. When all the samples have been collected from Steps 5 and 7, above, resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 µl of each sample on an SDS-PAGE gel and electrophorese. Store any remaining samples at -20°C.

*continued on next page*

# Expression of the PCR Product, continued

## Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µl of Bacterial Cell Lysis Buffer (see recipe on page 44).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times. Cells will easily lyse because some of the T7 lysozyme will leak out during the freeze-thaw cycle and digest the cell wall.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil for 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE gel and electrophorese. The SeeBlue® Plus2 Pre-Stained Standard (Catalog no. LC5925) is available from Invitrogen for use as a molecular weight protein marker.

## Analysis of Samples

1. Stain the gel with Coomassie® blue and look for a band of increasing intensity in the expected size range for the recombinant fusion protein. Use the uninduced culture as a negative control. To simplify Coomassie® staining, the SimplyBlue™ SafeStain (Catalog no. LC6050) is available from Invitrogen.
2. In addition, you may perform a western blot (see below) to confirm that the overexpressed band is your desired protein.
3. Use the positive control to confirm that growth and induction were performed properly. The pCR®T7/VP22-1 and pCR®T7/VP22-2 vectors should both produce a 23 kDa protein when induced with IPTG.

**Note**

Fusion of your PCR product with the truncated VP22 ORF and the C-terminal peptide will increase the size of your protein by approximately 24 kDa if you are using pCR®T7/VP22-1-TOPO® and 23 kDa if you are using pCR®T7/VP22-2-TOPO®.

## Western Blot Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine (6xHis) tag. The ready-to-use WesternBreeze™ Chromogenic Kits and WesternBreeze™ Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, visit www.invitrogen.com or call Technical Service (see page 48).

## The Next Step

If you are satisfied with expression of your gene of interest, proceed to purification, page 23.

If you have trouble expressing your protein, or wish to optimize expression, see the next page.

# Troubleshooting Expression

## Introduction

Information is provided below to help you troubleshoot your expression experiment.

## No Expression

- If you are cloning into pCR®T7/VP22-2-TOPO®, sequence your construct to make sure that your PCR product is in frame with the T7 leader.
- If you are cloning into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, sequence your construct to make sure it is in frame with the truncated VP22 ORF and the C-terminal peptide.
- If the positive control is expressed, but you don't see any expression from your construct on a Coomassie® stained gel, analyze your samples using Western blotting. Use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein.

## Low Expression

If your protein is expressed, but the levels are low, it is possible that expression of your gene may be toxic to *E. coli*. This is the most common reason for poor expression. Evidence of toxicity may include the following:

- Slow growth relative to the control
- Loss of plasmid

To reduce the toxicity of your gene, basal levels of T7 RNA polymerase must be reduced. There are a number of methods to reduce basal level expression of T7 RNA polymerase. The choice of method depends on the relative toxicity of your gene product to *E. coli*. The table below outlines the method choices.

| Relative Toxicity | Method | Comments |
|---|---|---|
| Moderate | Transformation into a pLysE-containing strain | Substantial levels of T7 lysozyme produced. Growth rate may be reduced. |
| High | Infect TOP10F′ (or any other suitable host strain) with M13 or lambda phage expressing T7 RNA polymerase | T7 RNA polymerase is not present in the cell until infection. Requires growth and maintenance of phage stocks. |

**Note**

Many researchers use the leakiness of the T7 system to their advantage. In some cases, basal level, constitutive expression produces sufficient protein for analysis and purification, particularly if the host strain containing the construct of interest is grown at room temperature. We recommend growing the strain for 24-48 hours at room temperature to produce sufficient protein. Expression of your construct using this method can result in substantial production of soluble protein.

**Tip:** To optimize production of soluble protein using the above method, try BL21(DE3) cells, which do not express T7 lysozyme.

*continued on next page*

# Troubleshooting Expression, continued

## Obtaining Other BL21 Strains

BL21(DE3) and BL21(DE3)pLysE cells are available from Invitrogen in the One Shot® format (see below). Visit www.invitrogen.com or contact Technical Service for more information (see page 48).

| Cells | Catalog no. |
|---|---|
| One Shot® BL21(DE3) | C6000-03 |
| One Shot® BL21(DE3)pLysE | C6565-03 |

**Note**

Do not use BL21(DE3), BL21(DE3)pLysS, or BL21(DE3)pLysE to propagate or maintain your pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® construct. Use TOP10 cells instead (see page 4).

## Infection with Phage

In about 5% of all cases, there will be some genes that are so toxic that they require infection with phage expressing T7 RNA polymerase (Tabor, 1990). You will need to use an *E. coli* host strain that contains the F′ episome (e.g. TOP10F′ or DH5αF′). Remember that the BL21(DE3)pLysS and BL21(DE3)pLysE strains should **not** be used in this situation. A protocol for infecting *E. coli* with M13 phage expressing T7 polymerase can be found in *Current Protocols in Molecular Biology*, pp. 16.2.1-16.2.11 (Ausubel *et al.*, 1994). Information for infecting *E. coli* with lambda phage expressing T7 polymerase is also available (Studier *et al.*, 1990). TOP10F′ cells are available from Invitrogen (Catalog no. C615-00) for use in phage infections.

# Purification of VP22 Fusion Proteins

## Introduction

Once you have expressed your recombinant fusion protein from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®, you are ready to scale-up expression for purification of the fusion protein from the bacterial culture. The presence of the C-terminal polyhistidine (6xHis) tag in your VP22 fusion protein allows you to use a metal-chelating resin such as ProBond™ to purify your fusion protein. After purification, the VP22 fusion protein can be applied directly to the mammalian cells of choice.

## Important

This section provides guidelines to purify your VP22 fusion protein using ProBond™. If you are using another metal-chelating resin, follow the manufacturer's instructions. Other purification methods are also suitable. Note that some empirical experimentation may be necessary to determine the optimum conditions required to purify your fusion protein.

## ProBond™ Resin

ProBond™ is a nickel-charged Sepharose® resin that can be used for affinity purification of fusion proteins containing the 6xHis tag. Proteins bound to the resin can be eluted by competition with imidazole. ProBond™ resin is available as part of the ProBond™ Purification System or in bulk from Invitrogen (see page ix for ordering information). For more information, visit www.invitrogen.com or call Technical Service (see page 48).

## Binding Capacity of ProBond™

The binding capacity of ProBond™ is approximately 1 mg of recombinant protein per milliliter of bed volume. Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein to the column.

We generally use 2 ml of ProBond™ resin to purify VP22 fusion proteins from 50 ml of bacterial culture. The amount of purified fusion protein obtained from 50 ml of bacterial culture is generally sufficient to treat up to 50 dishes of cells (plated in 35 mm dishes or tissue culture wells). Note that yields of purified recombinant protein will vary depending on the nature of the protein. If you need to purify larger amounts of recombinant protein, you may need more ProBond™ resin (see page ix for ordering information).

## Positive Control

We recommend that you also purify the control VP22 fusion protein from the pCR®T7/VP22-1 or pCR®T7/VP22-2 plasmid while purifying your recombinant fusion protein from pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO®. You may use the purified VP22 fusion protein (expressed from pCR®T7/VP22-1 or pCR®T7/VP22-2) as a control to assay uptake in your mammalian cell line of interest.

*continued on next page*

# Purification of VP22 Fusion Proteins, continued

## Materials Supplied by the User

You will need to have the following reagents and equipment on hand before beginning.

- One vial of One Shot® BL21(DE3)pLysS cells and transformation reagents for each expression experiment (included with the kit)
- pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® construct (and positive control)
- LB medium containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol
- 1 M IPTG
- 37°C shaking incubator
- VP22 Lysis Buffer (see page 45 for a recipe)
- VP22 Wash Buffer (see page 46 for a recipe)
- Three different VP22 Elution Buffers (100 mM Elution Buffer, 200 mM Elution buffer, and 500 mM Elution Buffer; see page 46 for a recipe)
- 0.5 M β-mercaptoethanol
- 1 mg/ml leupeptin (prepare in sterile water; store at –20°C)
- 1 mg/ml pepstatin (prepare in methanol; store at –20°C)
- 0.5 M phenyl methyl sulfonyl fluoride (PMSF; prepare in methanol)
- 100 mg/ml lysozyme (prepare in VP22 Lysis Buffer immediately before use)
- 10 mg/ml DNase I
- 10 mg/ml RNase A
- 21-gauge needles
- 5 ml syringes
- Sonicator
- ProBond™ (resin or prepacked columns)
- 15 ml and 50 ml sterile conical tubes
- 1.5 ml microcentrifuge tubes
- Appropriate equipment to hold ProBond™ column and collect fractions
- 1X and 4X SDS-PAGE sample buffer
- Reagents and apparatus for SDS-PAGE gel
- Boiling water bath

*continued on next page*

# Purification of VP22 Fusion Proteins, continued

### Scale-up of Expression for Purification on ProBond™

Use the procedure below to grow and induce expression of your VP22 fusion protein from 50 ml of bacterial culture. If you need to purify larger amounts of recombinant protein, you may scale-up the volume of bacterial culture accordingly.

1. Inoculate 10 ml of SOB or LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with a BL21(DE3)pLysS transformation reaction (see protocol on page 18).
2. Grow overnight at 37°C with shaking (225-250 rpm) to $OD_{600}$ = 1-2.
3. The next day, inoculate 50 ml of SOB or LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with 2 ml of the overnight culture. **Note:** You can scale up further and inoculate all of the 10 ml overnight culture into 500 ml of medium, but you will need a larger bed volume for your ProBond™ column.
4. Grow the culture at 37°C with shaking (225-250 rpm) to an $OD_{600}$ = ~0.5 (2-3 hours). The cells should be in mid-log phase. Remove a 500 μl aliquot of the culture and centrifuge at 10,000 x g for 2 minutes. Discard the supernatant and resuspend the pellet in 50 μl of 1X SDS/PAGE sample buffer. This will be your gel sample of uninduced cells.
5. Add IPTG to the remaining culture to a final concentration of 1 mM to induce expression.
6. Grow at 37°C with shaking until the optimal time point determined by the pilot expression is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. Proceed directly to **Preparation of Cell Lysate** (see the next page) or store the cell pellets at -80°C for future use. If you are using a metal-chelating resin other than ProBond™, refer to the manufacturer's instructions.

*continued on next page*

# Purification of VP22 Fusion Proteins, continued

## Preparing Cell Lysate

Before beginning, we recommend that you read through the protocol. Be sure to have all of your solutions prepared.

1. Add 4 ml of ice cold VP22 Lysis Buffer to each cell pellet from Step 6, previous page. To each 4 ml sample, add the following:

| | |
|---|---|
| 0.5 M β-mercaptoethanol | 40 µl |
| 1 mg/ml Leupeptin | 4 µl |
| 1 mg/ml Pepstatin | 4 µl |
| 0.5 M PMSF | 4 µl |
| 100 mg/ml Lysozyme | 40 µl |

2. Resuspend the cell pellet making sure the cell pellet is fully dispersed. Keep samples on ice.
3. Incubate the cell lysate on ice for 20 to 30 minutes.
4. Sonicate the cell lysate for 3 x 10 seconds. Keep the samples on ice.
5. Add DNase I and RNase A to a final concentration of 10 µg/ml each. Incubate on ice for 20 minutes.
6. Pass the cell lysate through a 21-gauge needle on a 5 ml syringe. Repeat twice.
7. Centrifuge the cell lysate at 20,000 x g for 15 minutes at +4°C.
8. Transfer the supernatant to a new tube. Remove and save an aliquot of the supernatant for later analysis. This sample contains your soluble fusion protein. Store samples at +4°C. For long-term storage, store the samples at –80°C. Proceed to **Purification on ProBond™**, next page.

   **Note:** If you wish to determine the amount of expressed fusion protein that is insoluble, resuspend the cell pellet in 4 ml of VP22 Lysis Buffer. Remove an aliquot of the lysate and save for later analysis.

*continued on next page*

# Purification of VP22 Fusion Proteins, continued

## Purification on ProBond™

To prepare buffers, see page 44. To purify your fusion protein using ProBond™ resin:

1. To equilibrate the ProBond™ resin, add the resin to a 2 ml column and add 10 ml of ice cold VP22 Lysis Buffer to the column. Cap the top of the column and place the column horizontally on ice. Rock gently on a shaking platform for 2-3 minutes. Attach the column vertically to a stand and allow the resin to settle. Carefully remove the Lysis Buffer by pipetting.
2. Apply the supernatant containing your soluble fusion protein from Step 8, previous page to the column. Cap the top of the column and place horizontally on ice. Place on a shaking platform and allow the resin and supernatant to mix for one to two hours at +4°C.
3. Clamp the column vertically and allow the resin to settle. Remove the caps from the top and then the bottom of the column and allow the supernatant to pass through the column. Collect the flow-through and remove an aliquot for later analysis. This sample contains unbound protein.
4. Add 10 ml of ice cold VP22 Lysis Buffer to the column. Collect the flow-through and remove an aliquot for later analysis.
5. Add 20 ml of ice cold VP22 Wash Buffer to the column. Collect the flow-through and remove an aliquot for later analysis.
6. To elute your recombinant fusion protein, use the three VP22 Elution Buffers that you have prepared. Apply 3 ml of ice cold 100 mM VP22 Elution Buffer to the column. Collect the flow-through in one tube. Remove an aliquot of the flow-through and save for later analysis.

   **Note:** Generally, the majority of the fusion protein will not elute from the ProBond™ column until the 500 mM VP22 Elution Buffer is used. This may vary depending on the nature of your protein.
7. Apply 3 ml of ice cold 200 mM VP22 Elution Buffer to the column. Collect the flow-through in one tube. Remove an aliquot of the flow-through and save for later analysis.
8. Apply 3 ml of ice cold 500 mM VP22 Elution Buffer to the column. Collect the flow-through as 0.5 ml fractions. These fractions should contain your eluted fusion protein. Remove an aliquot from each fraction for SDS-PAGE analysis.
9. Remove 10 µl of the resin from the column. Add 20 µl sterile water and 10 µl of 4X SDS-PAGE sample buffer (see recipe on page 45). This sample will contain protein that has not eluted from the resin.
10. For SDS-PAGE, add SDS-PAGE sample buffer to the various eluted fractions to a final concentration of 1X and heat the samples at 70ºC for 5 minutes. Load samples onto an SDS-PAGE gel and electrophorese. Stain gel with Coomassie® stain and identify the fraction containing the most concentrated eluted recombinant protein. If protein is not detectable by Coomassie® staining, you may want to perform Western blot analysis (see page 20) to gauge the success of your purification. To determine the concentration of your fusion protein, see the next page.
11. Once you have identified the fractions containing your eluted fusion protein, pool the fractions and store them at +4°C for immediate use. For long-term storage, store the purified protein at -80°C (see the next page).

*continued on next page*

# Purification of VP22 Fusion Proteins, continued

### Long-Term Storage of Purified Fusion Protein

We have stored purified VP22 fusion proteins at -80°C for up to 4 weeks, therefore, it may be possible to store your purified fusion protein at -80°C. Storage conditions may vary depending on the nature of your protein. Note that freezing your purified fusion protein may affect its biological activity. If you plan to store your purified fusion protein at -80°C, avoid repeated freezing and thawing as it may result in loss of activity or protein integrity.

**Note**

We have found that the degree of purification achieved for VP22 fusion proteins using ProBond™ is sufficient for most applications in mammalian cells. In general, no further purification steps are required. This may vary depending on the nature of your protein and your specific application.

### Determining Fusion Protein Concentration

You may use any method of your choice to determine the concentration of your purified fusion protein. We typically use the following techniques to determine the concentration of purified VP22 fusion proteins.

1. For a rough estimate of fusion protein concentration, run samples of the purified fusion protein and appropriate standards containing known amounts of protein (e.g. BSA) on an SDS/PAGE gel. Stain the gel with Coomassie™ blue or Simply Blue™ Safe Stain and use the standards to estimate the concentration of your fusion peptide.
2. To more accurately determine your fusion peptide concentration, we recommend using an assay that tolerates the presence of imidazole in the sample. We do not recommend performing Bradford or Lowry assays because the presence of imidazole may interfere with accurate protein concentration determination.

### Yield of Purified Fusion Protein

When purifying VP22 fusion proteins using the protocol on pages 26-27, we generally obtain 1-10 mg of purified protein at a concentration ranging from 1-10 µg/µl. Note that protein yields will vary depending on the nature of the protein and its solubility. We have found that the purified fusion protein is generally concentrated enough to add directly to mammalian cells and no further concentration is required. If you need a more concentrated protein solution, refer to general references (Coligan *et al.*, 1998; Deutscher, 1990) for guidelines and protocols to help you concentrate your fusion protein.

### Amount of Fusion Protein to Add to Mammalian Cells

We typically add 1-10 µg of purified fusion protein to 2 x $10^5$ cells plated in a 35 mm dish or tissue culture well. Larger amounts of purified fusion protein may be added, if desired. The amount of purified fusion protein you need to add to your mammalian cells may vary depending on the nature of your protein of interest and your application.

# Application and Detection of Purified Protein in Mammalian Cells

## Introduction

Once you have obtained your purified VP22 fusion protein, you are ready to add the purified fusion protein to cultured mammalian cells. The section below provides information on application and detection of your fusion protein in mammalian cells.

## Detection of Fusion Protein

Before plating cells, you need to determine the technique that you will use to assay for uptake of your fusion protein (e.g. immunofluorescence, western analysis). Once you have designed your experiment, seed your cells accordingly.

To detect the VP22 fusion protein in mammalian cells, you may use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein. The Anti-*myc* antibodies and the Anti-His(C-term) antibodies can be used to detect your protein using immunofluorescence or western blotting.

Be sure to include a sample of purified control VP22 fusion protein (expressed from the parental pCR®T7/VP22-1 or pCR®T7/VP22-2 vector) to help you evaluate your results. The control VP22 fusion protein serves as a positive control for translocation and as a negative control for potential biological effects observed with your fusion protein. Also include a sample of 500 mM VP22 elution buffer containing no protein as a negative control for uptake and biological effects observed with your fusion protein.

**Important**

The presence of serum in the medium can inhibit uptake of VP22 fusion proteins into mammalian cells. You will need to **remove** the serum-containing medium from your cells and replace with medium containing no serum immediately prior to addition of purified fusion protein. Since uptake occurs within 20 minutes after addition of VP22 fusion protein to the cells, we have not observed any deleterious phenotypic effects caused by the short-term removal of serum in the cell lines tested. You may want to keep this in mind if your cells are particularly sensitive to removal of serum.

## Experimental Outline

The table below outlines the basic steps needed to add and detect VP22 fusion proteins in your mammalian cells of interest.

| Step | Action |
|---|---|
| 1 | Decide on the technique that you will use to assay for uptake of your VP22 fusion protein and seed your mammalian cells accordingly. Incubate cells overnight at 37°C. |
| 2 | The next day, remove serum-containing medium from the cells and replace with medium containing no serum. |
| 3 | Add purified VP22 fusion protein or control fusion protein to the cells. |
| 4 | Incubate cells at 37°C for 20 minutes. |
| 5 | Assay for uptake of your fusion protein by immunofluorescence, western blot, or other method of your choice. |

*continued on next page*

# Application and Detection of Purified Protein in Mammalian Cells, continued

**Note**

You will be adding purified fusion protein directly to the culture medium of your mammalian cells. Remember that your protein solution contains imidazole. Although we have not observed any pleiotrophic effects of imidazole on cells, this may vary depending on the nature of your cell line. If the presence of imidazole is a concern, you may dialyze your fusion protein solution to remove the imidazole. **When applying the VP22 fusion protein to mammalian cells, we recommend that the amount of VP22 fusion protein solution added to the culture medium not exceed 5% of the culture volume.**

## Immuno-fluorescence

A sample immunofluorescence protocol is provided below using the Anti-*myc* Antibody. Other protocols and antibodies (see below) may be suitable.

1. Plate cells (~$10^5$) in a 35 mm dish or a single well in a six-well tissue culture plate. Incubate cells overnight at 37°C in serum-containing medium.
2. The next day, remove the serum-containing medium and replace with medium containing no serum (1 ml for a 35 mm dish or a single well in a six-well tissue culture plate) immediately before addition of the purified VP22 fusion protein.
3. Add the appropriate amount of VP22 fusion protein directly to the medium of the cells. Swirl gently to mix. Incubate at 37°C for 20 minutes.
4. Remove the medium and wash cells twice with PBS (Phosphate buffered saline; Catalog no. 10010-023). Fix the cells by adding 2 ml of room temperature, 100% methanol. **Note**: The best results have been obtained with fixation using methanol rather than formaldehyde.
5. Incubate for 5 minutes at room temperature. Do not exceed 5 minutes.
6. After incubation, wash cells 5 times with PBS (2 ml/wash).
7. Add 2 ml PBS containing 10% fetal bovine serum (FBS) (blocking solution) and incubate for 15 minutes at room temperature to reduce non-specific binding of antibody.
8. Remove the blocking solution and add 1 ml of PBS/10% FBS containing the Anti-*myc* Antibody (1:500 dilution of antibody). Incubate for 20 minutes at room temperature.
9. Wash cells 2 x 5 minutes with PBS.
10. Dilute goat anti-mouse Oregon Green conjugate (Catalog no. O-6383) 1:500 in PBS/10% FBS. **Note**: Other fluorescent conjugates can be used.
11. Add to cells and incubate for 20 minutes at room temperature in the dark.
12. Wash cells 2 x 5 minutes with PBS and observe cells with fluorescence microscope equipped with a FITC filter (or appropriate filter).

For your convenience, FITC-conjugated antibodies are available from Invitrogen (see page ix for ordering information) to facilitate direct immunofluorescence detection of VP22 fusion proteins without the need for secondary fluorescent conjugates. If you are using the Anti-*myc*-FITC Antibody or Anti-His(C-term)-FITC Antibody for immunofluorescence, refer to the FITC-conjugated Antibodies manual for instructions for use.

*continued on next page*

# Application and Detection of Purified Protein in Mammalian Cells, continued

## What You Should See

Cells that have taken up the native VP22 protein typically show nuclear staining (Brewis *et al.*, 2000; Elliott and O'Hare, 1997; Phelan *et al.*, 1998). If you have included the control VP22 fusion protein (expressed from pCR®T7/VP22-1) in your experiment, the cells should also show nuclear staining.

In experiments at Invitrogen, we have seen both nuclear and cytoplasmic staining in mammalian cells which have taken up recombinant VP22 fusion proteins. The localization of the fusion protein appears to be determined, in part, by the nature of the protein of interest. We have TOPO® Cloned the human *rhoA* gene into pCR®T7/VP22-1-TOPO® and purified bacterially-expressed VP22-rhoA fusion protein. The rhoA protein is generally found in the cytoplasm of mammalian cells where it is involved in the polymerization of actin microfilaments (Hall, 1998). When applied to murine Swiss 3T3 cells, biologically active VP22-rhoA fusion protein localizes to the cytoplasm of recipient cells. In contrast, a biologically active fusion protein composed of VP22 and the Green Fluorescent Protein (GFP) localizes predominantly to the nucleus of rat PC12 and human Jurkat cells.

## No Fluorescence

Assuming the VP22 fusion protein has been imported into the cells, review the immunofluorescence procedure.

- Try a different fixation method
- Try a different antibody

## Preparing Cell Lysates for Western Blot

To detect your VP22 fusion protein by western blot, you will need to prepare a cell lysate from treated cells. A sample protocol is provided below. Other protocols and lysis buffers may be suitable.

1. Seed cells in 35 mm culture dishes at 50% confluency (approximately 1 x $10^5$ cells) in 2 ml of culture medium. Allow cells to adhere overnight.
2. The next day, remove serum-containing medium from the cells and replace with 1.5 ml of medium containing no serum.
3. Add the appropriate amount of VP22 fusion protein directly to the culture medium. Swirl gently to mix. Incubate cells at 37°C for 20 minutes.
4. Wash cell monolayer once with phosphate-buffered saline (PBS) at pH 7.4 .
5. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
6. Resuspend in 50 µl Mammalian Cell Lysis Buffer (see page 47 for a recipe). Other cell lysis buffers are suitable. Vortex.
7. Incubate cell suspension at 37°C for 10-15 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
8. Centrifuge the cell lysate at 10,000 x g for 5-10 minutes at room temperature to pellet nuclei and transfer the supernatant to a fresh tube.
9. Add SDS-PAGE sample buffer to a final concentration of 1X and heat the sample at 70ºC for 5 minutes. Load 20 µl onto an SDS-PAGE gel and electrophorese. Perform western blot analysis using your desired protocol.

# Appendix

## pCR®T7/VP22 TOPO TA Cloning® Control Reactions

### Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing kanamycin and X-gal.

### Before Starting

Be sure to prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page 44 for recipe)
- LB plates containing 50 µg/ml kanamycin and X-gal (two per transformation)
- To add X-gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

### Producing Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and *LacZα*, set up the following 50 µl PCR:

| | |
|---|---|
| Control DNA Template (50 ng) | 1 µl |
| 10X PCR Buffer | 5 µl |
| 50 mM dNTPs | 0.5 µl |
| Control PCR Primers (0.1 µg/µl) | 1 µl |
| Sterile Water | 41.5 µl |
| *Taq* Polymerase (1 unit/µl) | 1 µl |
| Total Volume | 50 µl |

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

| Step | Time | Temperature | Cycles |
|---|---|---|---|
| Initial Denaturation | 2 minutes | 94°C | 1X |
| Denaturation | 1 minute | 94°C | 25X |
| Annealing | 1 minute | 60°C | |
| Extension | 1 minute | 72°C | |
| Final Extension | 7 minutes | 72°C | 1X |

4. **Remove** 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

*continued on next page*

# pCR®T7/VP22 TOPO TA Cloning® Control Reactions, continued

## Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

| Reagent | "Vector Only" | "Vector + PCR Insert" |
|---|---|---|
| Control PCR Product | -- | 1 µl |
| Salt Solution or Dilute Salt Solution | 1 µl | 1 µl |
| Sterile Water | 4 µl | 3 µl |
| TOPO® vector | 1 µl | 1 µl |

2. Incubate at 25°C (room temperature) for 5 minutes and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (page 13).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin and X-gal. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

## Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 85% of these will be blue and contain the 500 bp insert.

The 'vector only' plate should contain only a few colonies (<15% of the vector + PCR insert plate).

## Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 13. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 50 µg/ml ampicillin. Transformation efficiency should be ~1 x $10^9$ cfu/µg DNA.

*continued on next page*

# pCR®T7/VP22 TOPO TA Cloning® Control Reactions, continued

## Factors Affecting Cloning Efficiency

Note that lower transformation and/or cloning efficiencies will result from the following variables. Most of these are easily corrected, but if you are cloning large inserts, you may not obtain the expected 85% cloning efficiency.

| Variable | Solution |
|---|---|
| pH>9 | Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8. |
| Incomplete extension during PCR | Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time. |
| Cloning large inserts (>3 kb) | Try one or all of the following:<br>Increase amount of insert.<br>Incubate the TOPO® Cloning reaction longer.<br>Gel-purify the insert as described on pages 35-36. |
| Excess (or overly dilute) PCR product | Reduce (or concentrate) the amount of PCR product. **Note**: You may use up to 4 µl of your PCR in the TOPO® Cloning reaction. |
| Cloning blunt-ended fragments | Add 3´ A-overhangs by incubating with *Taq* polymerase (page 37). |
| PCR cloning artifacts ("false positives") | TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (pages 35-36) or optimize your PCR. |
| PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase | *Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996). |

# Purifying PCR Products

## Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below for your convenience.

**Note**

Note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 10).

## Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 13.

## Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 13). Be sure to make the gel slice as small as possible for best results.

*continued on next page*

# Purifying PCR Products, continued

### Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 13.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes.** This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 13.

**Note**

Note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

# Addition of 3´ A-Overhangs Post-Amplification

## Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the lack of the terminal transferase activity associated with proofreading polymerases which adds the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

## Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

## Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
Incubate at 72°C for 8-10 minutes (do not cycle).
Place the vials on ice. The DNA amplification product is now ready for ligation into the TOPO® vector.
**Note:** If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

**Note**

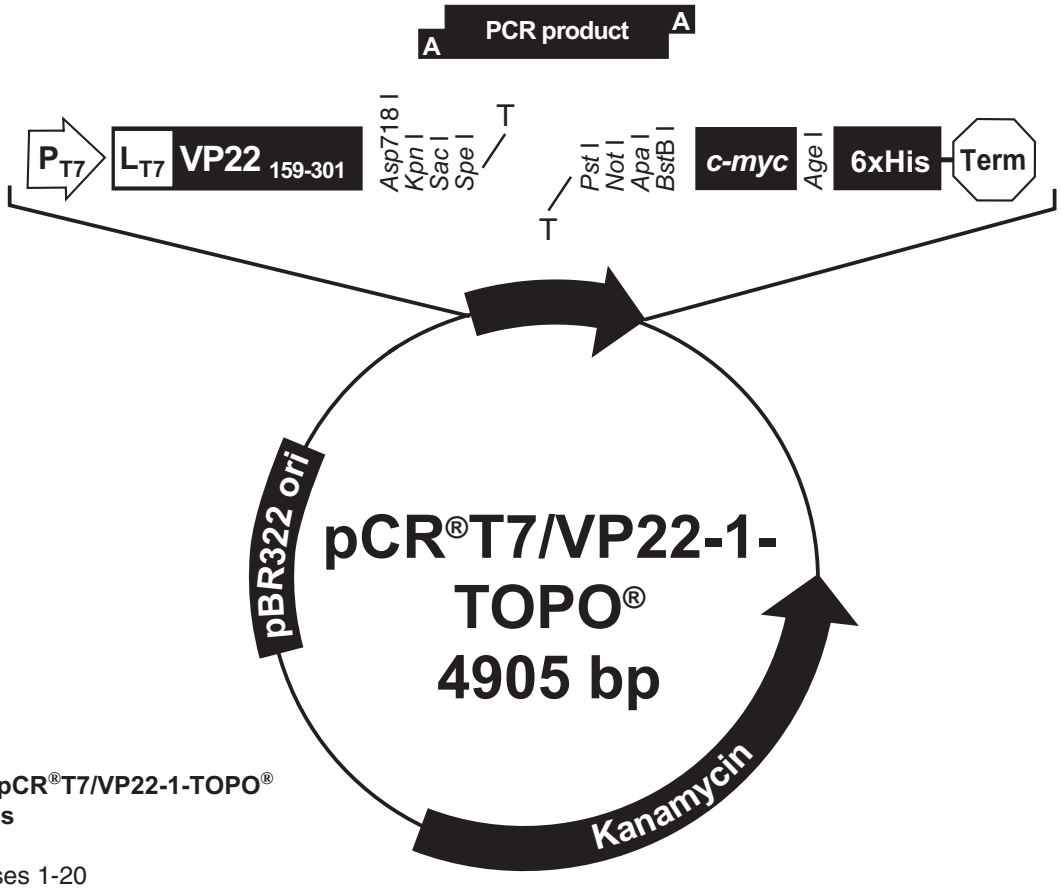
You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see previous page). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

Vent® is a registered trademark of New England Biolabs.

# pCR®T7/VP22-1-TOPO®

## Map

The figure below summarizes the features of the pCR®T7/VP22-1-TOPO® vector. The vector is supplied linearized between base pairs 597 and 598. This is the TOPO® Cloning site. **The complete nucleotide sequence for pCR®T7/VP22-1-TOPO® is available for downloading from www.invitrogen.com or from Technical Service (see page 48).**




**Comments for pCR®T7/VP22-1-TOPO®**
**4905 nucleotides**

T7 promoter: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Truncated VP22 ORF (amino acids 159-301 only): bases 126-551
VP22 forward priming site: bases 455-470
TOPO® Cloning site: bases 597-598
*c-myc* epitope: bases 661-690
Polyhistidine (6xHis) tag: bases 706-723
*myc*-His reverse priming site: bases 688-708
Kanamycin resistance gene: bases 1199-2014 (complementary strand)
pBR322 origin: bases 2110-2783

*continued on next page*

# pCR®T7/VP22-1-TOPO®, continued

## Features

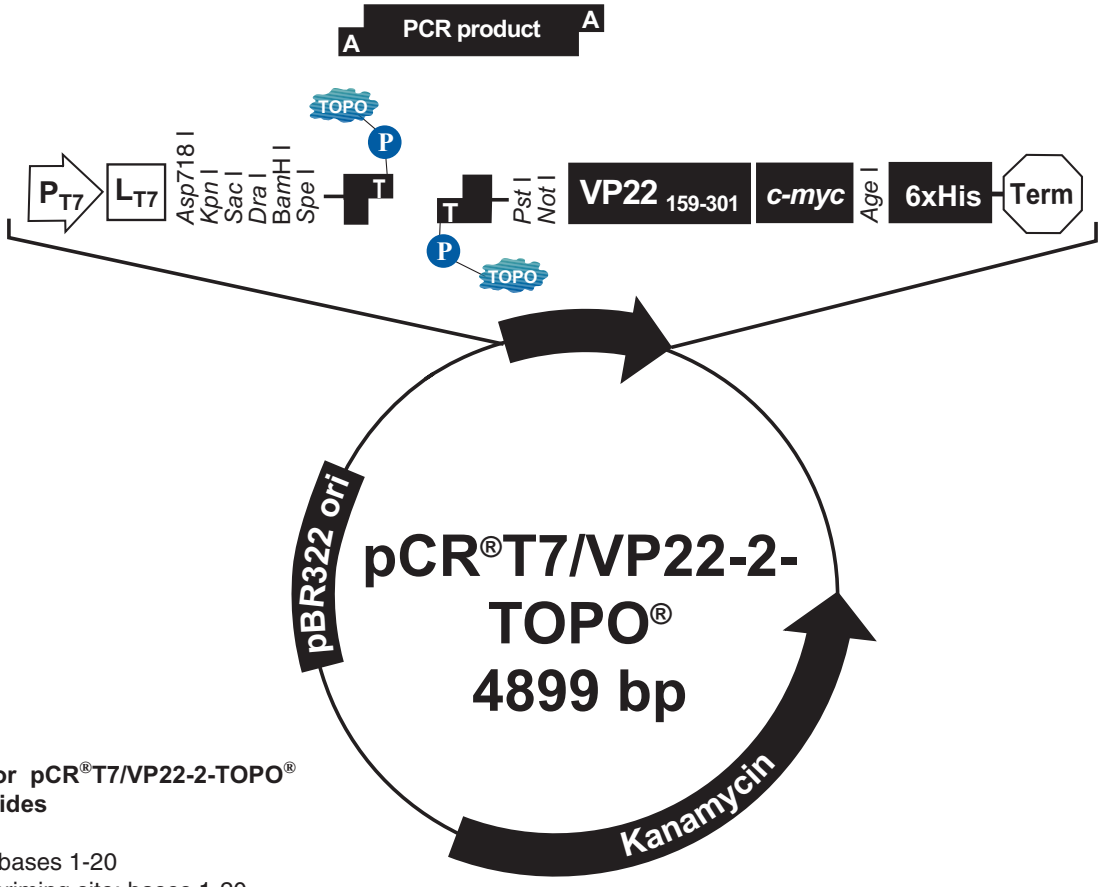
The pCR®T7/VP22-1-TOPO® vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

| Feature | Benefit |
|---|---|
| T7 promoter | Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase |
| T7 leader | Improves translation efficiency of the gene of interest |
| Truncated VP22 ORF (amino acids 159-301 only) | Fusing your gene to the truncated VP22 ORF permits translocation of the protein into mammalian cells (Elliott and O'Hare, 1997) |
| VP22 Forward priming site | Permits sequencing of your insert to confirm that it is in frame with the truncated VP22 ORF |
| TOPO® Cloning site | Allows insertion of your PCR product in frame with the truncated VP22 ORF and the C-terminal peptide containing the *c-myc* epitope and polyhistidine tag |
| *c-myc* epitope<br>(Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) | Allows detection of your recombinant protein with the Anti-*myc* Antibody (Catalog no. R950-25) or Anti-*myc*-HRP Antibody (Catalog no. R951-25) (Evans *et al.*, 1985) |
| C-terminal polyhistidine (6xHis) tag | Permits purification of your recombinant VP22 fusion protein on metal-chelating resin such as ProBond™<br>In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25) (Lindner *et al.*, 1997) |
| *myc*-His Reverse priming site | Permits sequencing of your insert |
| Kanamycin resistance gene | Allows selection of the vector in *E. coli* |
| pBR322 origin | Permits replication and low-copy maintenance of the plasmid in *E. coli* |

# pCR®T7/VP22-2-TOPO®

## Map

The figure below summarizes the features of the pCR®T7/VP22-2-TOPO® vector. The vector is supplied linearized between base pairs 180 and 181. This is the TOPO® Cloning site. **The complete nucleotide sequence for pCR®T7/VP22-2-TOPO® is available for downloading from www.invitrogen.com or from Technical Service (see page 48).**




**Comments for pCR®T7/VP22-2-TOPO®**
**4899 nucleotides**

T7 promoter: bases 1-20
T7 promoter/priming site: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
TOPO® Cloning site: bases 180-181
Truncated VP22 ORF (amino acids 159-301 only): bases 226-651
VP22 reverse 2 priming site: bases 273-288
*c-myc* epitope: bases 655-684
Polyhistidine (6xHis) tag: bases 700-717
Kanamycin resistance gene: bases 1193-2008 (complementary strand)
pBR322 origin: bases 2104-2777

*continued on next page*

# pCR®T7/VP22-2-TOPO®, continued

## Features

The pCR®T7/VP22-2-TOPO® vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

| Feature | Benefit |
|---|---|
| T7 promoter/priming site | Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase and allows sequencing of your insert |
| T7 leader | Improves translation efficiency of the gene of interest |
| TOPO® Cloning site | Allows insertion of your PCR product in frame with the truncated VP22 ORF and the C-terminal peptide containing the *c-myc* epitope and polyhistidine tag |
| Truncated VP22 ORF (amino acids 159-301 only) | Fusing your gene to the truncated VP22 ORF permits translocation of the protein into mammalian cells (Elliott and O'Hare, 1997) |
| VP22 Reverse 2 priming site | Permits sequencing of your insert |
| *c-myc* epitope<br>(Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) | Allows detection of your recombinant protein with the Anti-*myc* Antibody (Catalog no. R950-25) or Anti-*myc*-HRP Antibody (Catalog no. R951-25) (Evans *et al.*, 1985) |
| C-terminal polyhistidine (6xHis) tag | Permits purification of your recombinant VP22 fusion protein on metal-chelating resin such as ProBond™<br>In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25) (Lindner *et al.*, 1997) |
| Kanamycin resistance gene | Allows selection of the vector in *E. coli* |
| pBR322 origin | Permits replication and low-copy maintenance of the plasmid in *E. coli* |

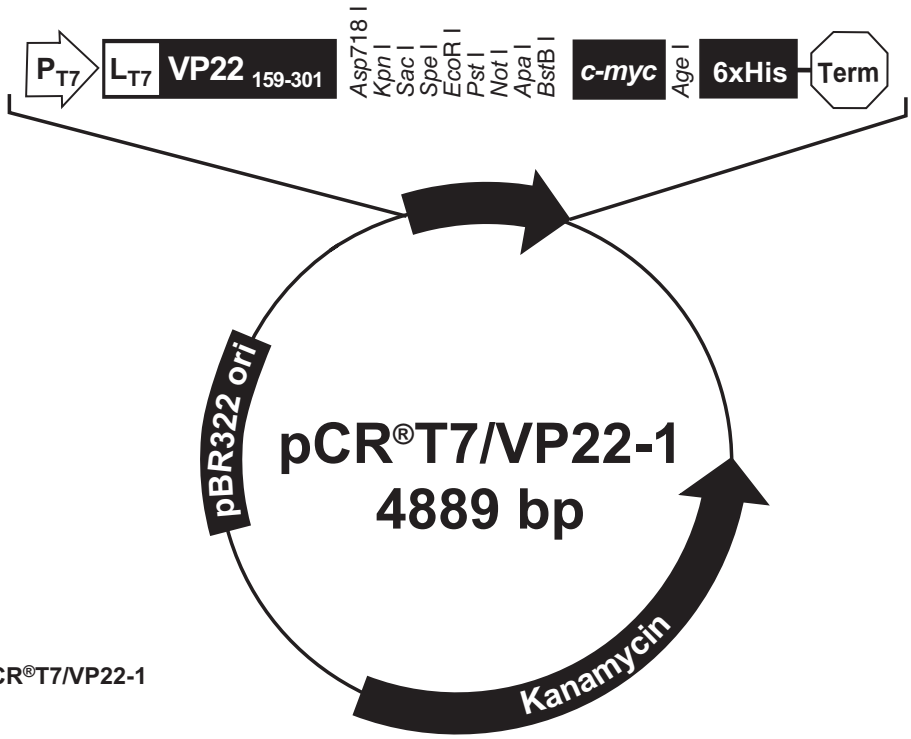
# Map of pCR®T7/VP22-1

## Description

pCR®T7/VP22-1 is a 4889 bp control vector containing the truncated VP22 ORF (amino acids 159-301) fused to the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. pCR®T7/VP22-1 is the parent vector of pCR®T7/VP22-1-TOPO® and may be used as a positive expression control. The recombinant VP22 fusion protein produced from pCR®T7/VP22-1 is 23 kDa in size. **Note**: The multiple cloning site maintains the frame through the C-terminal tag.

## Map of Control Vector

The figure below summarizes the features of the pCR®T7/VP22-1 vector. **The complete nucleotide sequence for pCR®T7/VP22-1 is available for downloading from our www.invitrogen.com or by contacting Technical Service (see page 48).**




**Comments for pCR®T7/VP22-1**
**4889 nucleotides**

T7 promoter: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Truncated VP22 ORF (amino acids 159-301 only): bases 126-551
VP22 forward priming site: bases 455-470
Multiple cloning site: bases 552-647
*c-myc* epitope: bases 645-674
Polyhistidine (6xHis) tag: bases 690-707
*myc*-His reverse priming site: bases 672-692
Kanamycin resistance gene: bases 1183-1998 (complementary strand)
pBR322 origin: bases 2094-2767

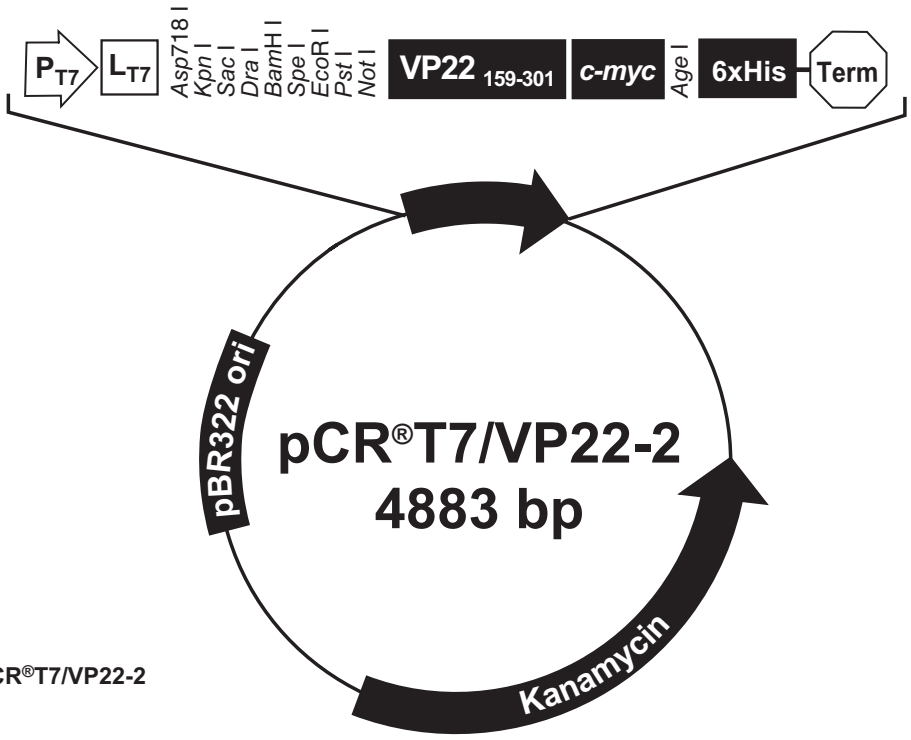
# Map of pCR®T7/VP22-2

## Description

pCR®T7/VP22-2 is a 4883 bp control vector containing the truncated VP22 ORF (amino acids 159-301) fused to the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. pCR®T7/VP22-2 is the parent vector of pCR®T7/VP22-2-TOPO® and may be used as a positive expression control. The recombinant VP22 fusion protein produced from pCR®T7/VP22-2 is 23 kDa in size. **Note**: The multiple cloning site maintains the frame through the C-terminal tag.

## Map of Control Vector

The figure below summarizes the features of the pCR®T7/VP22-2 vector. **The complete nucleotide sequence for pCR®T7/VP22-2 is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 48).**




**Comments for pCR®T7/VP22-2**
**4883 nucleotides**

T7 promoter: bases 1-20
T7 promoter/priming site: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Multiple cloning site: bases 123-203
Truncated VP22 ORF (amino acids 159-301 only): bases 210-635
VP22 reverse 2 priming site: bases 257-272
*c-myc* epitope: bases 639-668
Polyhistidine (6xHis) tag: bases 684-701
Kanamycin resistance gene: bases 1177-1992 (complementary strand)
pBR322 origin: bases 2088-2761

# Recipes

## LB (Luria-Bertani) Medium and Plates

**Composition:**

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (50 μg/ml kanamycin) if needed.
Store at room temperature or at +4°C.

**LB agar plates**

Prepare LB medium as above, but add 15 g/L agar before autoclaving.
Autoclave on liquid cycle for 20 minutes at 15 psi.
After autoclaving, cool to ~55°C, add antibiotic (50 μg/ml of kanamycin), and pour into 10 cm plates.
Let harden, then invert and store at +4°C, in the dark.

## X-Gal Stock Solution

To make a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethylformamide.
Protect from light by storing in a brown bottle at -20°C.
To add to previously made agar plates, warm the plate to 37°C. Pipette 40 μl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

## Bacterial Cell Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

1. Prepare 1 M stock solutions of $KH_2PO_4$ and $K_2HPO_4$.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   0.3 ml of 1 M $KH_2PO_4$
   4.7 ml of 1 M $K_2HPO_4$
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

*continued on next page*

# Recipes, continued

### 0.1 M Sodium Phosphate, pH 8.0

Before beginning, have the following reagents on hand.

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$, Sigma, Catalog no. S9638)
Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$, Sigma, Catalog no. S9390)

1. Prepare 100 ml of 1 M $NaH_2PO_4 \cdot H_2O$ by dissolving 13.8 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
2. Prepare 100 ml of 1 M $Na_2HPO_4 \cdot 7H_2O$ by dissolving 26.81 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
3. For 1 liter of 0.1 M sodium phosphate, pH 8.0, mix together 6.8 ml of 1 M $NaH_2PO_4 \cdot H_2O$ and 93.2 ml of 1 M $Na_2HPO_4 \cdot 7H_2O$. Bring volume up to 1 liter with sterile water. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at room temperature.

### VP22 Lysis Buffer

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
5 mM Imidazole (Sigma, Catalog no. I0125)

1. To 150 ml of deionized water, add the following and dissolve:

   250 ml of 0.1 M Sodium phosphate, pH 8.0 (see recipe above)
   8.77 g NaCl
   0.17 g Imidazole
2. Bring the volume up to 500 ml with deionized water.
3. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at +4°C.

*continued on next page*

# Recipes, continued

## VP22 Wash Buffer

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
40 mM Imidazole (Sigma, Catalog no. I0125)
10% glycerol

1. To 150 ml of deionized water, add the following and dissolve:

   250 ml of 0.1 M Sodium phosphate, pH 8.0 (see recipe on the previous page)
   8.77 g NaCl
   0.17 g Imidazole
   50 ml glycerol
2. Bring the volume up to 500 ml with deionized water.
3. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at +4°C.

## VP22 Elution Buffers

**Note that three different Elution Buffers should be prepared (100 mM Elution Buffer, 200 mM Elution Buffer, and 500 mM Elution Buffer).** The three Elution Buffers contain varying amounts of imidazole (see below).

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
10% glycerol
Imidazole (100 mM, 200 mM or 500 mM; Sigma, Catalog no. I0125)

1. To prepare the VP22 Elution Buffers, add the following to three separate sterile beakers and mix until dissolved:

| Reagent | 100 mM Elution Buffer | 200 mM Elution Buffer | 500 mM Elution Buffer |
|---|---|---|---|
| 0.1 M Sodium phosphate, pH 8.0 | 100 ml | 100 ml | 100 ml |
| NaCl | 3.51 g | 3.51 g | 3.51 g |
| Glycerol | 20 ml | 20 ml | 20 ml |
| Imidazole | 1.36 g | 2.72 g | 6.8 g |
| Deionized water | Volume up to 200 ml | Volume up to 200 ml | Volume up to 200 ml |

2. Check to make sure the pH is 8.0.
3. Filter-sterilize and store at +4°C.

*continued on next page*

# Recipes, continued

### Mammalian Cell Lysis Buffer

50 mM Tris-HCl, pH 7.8
150 mM NaCl
1% Nonidet P-40

This solution can be prepared from the following common stock solutions. For 100 ml, combine:

| | |
|---|---|
| 1 M Tris base | 5 ml |
| 5 M NaCl | 3 ml |
| Nonidet P-40 | 1 ml |

Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl. Bring the volume up to 100 ml. Store at room temperature.

**Note:** Just before use, add protease inhibitors to a small volume of lysis buffer at the following final concentrations:

1 mM PMSF
1 μg/ml pepstatin
1 μg/ml leupeptin

# Technical Service

## World Wide Web

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

## Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

**Corporate Headquarters:**
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

**Japanese Headquarters:**
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

**European Headquarters:**
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

## MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

## Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, report it to our Technical Service Representatives.

**Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.**

# Purchaser Notification

## Introduction

Use of the Voyager™ Protein Production Kits is covered under a number of different licenses including those detailed below.

## Information for European Customers

The One Shot® BL21(DE3)pLysS *E. coli* strain is genetically modified and carries a chromosomal insertion of a cassette containing the T7 RNA polymerase (T7 RNAP) gene. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

## Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.
Email: outlicensing@invitrogen.com

## Limited Use Label License No. 22: Vectors and Clones Containing Sequences Coding for Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

*continued on next page*

# Purchaser Notification, continued

**Limited Use Label License No: 30 T7 Expression System**

The composition and/or use of this product may be claimed in U.S. Patent No. 5,693,489 licensed to Life Technologies Corporation by Brookhaven Science Associates, LLC. The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy, and is the subject of patents and patent applications assigned to Brookhaven Science Associates, LLC (BSA,). By provisions of the Distribution License Agreement granted to Life Technologies covering said patents and patent applications, Life Technologies grants you a non-exclusive sub-license under patents assigned to BSA for the use of this technology, including the enclosed materials, based upon the following conditions: 1 – these materials are to be used for non-commercial research purposes only. A separate license under patents owned by BSA is required for any commercial use, including the use of these materials for research purposes or production purposes by any commercial entity. Information about commercial license may be obtained from The Office of Technology Transfer, Brookhaven National Laboratory, Bldg. 475D, P.O. Box 5000, Upton, New York 11973-5000. Phone (516) 344-7134. 2 - No materials that contain the cloned copy of the T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this sub-license and agrees to be bound by its terms. This limitation applies to strains BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE, CE6, BL21-SI Competent Cells and any derivatives that are made of them. You may refuse this sub-license by returning this product unused in which case Life Technologies accept return of the product with a full refund. By keeping or using this product, you agree to be bound by the terms of this license.

*Continued on next page*

# Purchaser Notification, continued

**Limited Use Label License No. 84: VP22 Technology**

This product is the subject of U.S. Patent No. 6,184,038 and foreign equivalents licensed exclusively to Invitrogen. The herpes simplex virus-1 structural protein VP22 was identified and characterized by scientists at Phogen Ltd., Cambridge, England. The product of the VP22 gene has been shown to have several unique properties including the ability to translocate into mammalian cells. The gene has been incorporated into a variety of products to facilitate its use by researchers. Invitrogen has an exclusive license to sell VP22-related products to scientists for research purposes only under the terms described below. Use of VP22 for any commercial purpose requires a commercial license as detailed below. Before using any VP22-related product, read the following license agreement. If you do not agree to be bound by its terms, contact Invitrogen within 10 days for authorization to return the unused VP22-related product and to receive a full refund. If you do agree to the terms of the Agreement, complete the Product User Registration Card and return it to Invitrogen before using the product.
Invitrogen Corporation (Invitrogen) grants you a non-exclusive sublicense to use the enclosed materials including the VP22 gene, protein product and/or antibody(ies) (collectively the "Materials") for research or for evaluation purposes only. The Materials are being transferred to you in furtherance of, and reliance on, such sublicense. You may not use the Materials for any commercial purpose without a license for such purpose from: Phogen, Attn: Business Development, 155 Cambridge Science Park, Cambridge CB4 4GN, England. Fax: +44-1223-426-115
Commercial purposes include, without limitation, the use or sale of VP22 or the Materials in a commercial product or process, in manufacturing, or in iterative research process(es) for commercial entities, e.g. iterative screening of compounds that are product or product intermediate candidates, or iterative use in creating or isolating templates for products.
Access to the Materials must be limited solely to those officers, employees and students of your institution who need access thereto in order to perform the above-described research or evaluation. You must inform each of such officer, employee and student of the provisions of this Agreement and require them to agree, in writing, to be bound by the provisions of this Agreement. You may not distribute the Materials to others, even those within your own institution. You may transfer modified or altered Materials to a third party following notification of Invitrogen such that the recipient can be licensed. You may not assign, sub-license, rent, lease or otherwise transfer this License or any of the rights or obligation hereunder, except as expressly permitted.
This license is effective until terminated. You may terminate it at any time by destroying all Materials (including altered and modified Materials) in your control. It will also terminate automatically if you fail to comply with the terms and conditions of this Limited Use Label License. You shall, upon termination of the license, notify Invitrogen in writing of such termination and execute a declaration that all Materials have been destroyed as outlined above.

# Product Specifications

## Introduction

This section describes the criteria used to qualify the components in the Voyager™ Protein Production Kits.

## Vectors

The pCR®T7/VP22-1 and pCR®T7/VP22-2 plasmids (parental vectors of pCR®T7/VP22-1-TOPO® and pCR®T7/VP22-2-TOPO®, respectively) are qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

## TOPO® Cloning Efficiency

Once the supercoiled vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 32-33, a 500 bp control PCR product is TOPO®-Cloned into pCR®T7/VP22-1-TOPO® or pCR®T7/VP22-2-TOPO® and subsequently transformed into the One Shot® TOP10 competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency.

## Sequencing Primers

The sequencing primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

## One Shot® Chemically Competent *E. coli*

All competent cells are tested for transformation efficiency using the control plasmid included in the One Shot® kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:

- Greater than 1 x $10^9$ cfu/µg plasmid DNA for TOP10
- Greater than 1 x $10^8$ cfu/µg plasmid DNA for BL21(DE3)pLysS

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

# References


Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Brewis, N., Phelan, A., Webb, J., Drew, J., Elliott, G., and O'Hare, P. (2000). Evaluation of VP22 Spread in Tissue Culture. J. Virol. *74*, 1051-1056.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996). Modulation of Non-Templated Nucleotide Addition by *Taq* DNA Polymerase: Primer Modifications that Facilitate Genotyping. BioTechniques *20*, 1004-1010.

Chang, A. C. Y., and Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. *134*, 1141-1156.

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1998). Current Protocols in Protein Science (New York: John Wiley).

Deutscher, M. P. (1990) Guide to Protein Purification. In *Methods in Enzymology*, Vol. 182. (J. N. Abelson and M. I. Simon, eds.) Academic Press, San Diego, CA.

Elliott, G., and O'Hare, P. (1997). Intercellular Trafficking and Protein Delivery by a Herpesvirus Structural Protein. Cell *88*, 223-233.

Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, V. M. (1985). Isolation of Monoclonal Antibodies Specific for *c-myc* Proto-oncogene Product. Mol. Cell. Biol. *5*, 3610-3616.

Hall, A. (1998). Rho GTPases and the Actin Cytoskeleton. Science *279*, 509-514.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. S. (1990) PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Phelan, A., Elliott, G., and O'Hare, P. (1998). Intercellular Delivery of Functional p53 by the Herpesvirus Protein VP22. Nature Biotechnology *16*, 440-443.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. J. Biol. Chem. *269*, 32678-32684.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA Polymerase to Direct Expression of Cloned Genes. Methods in Enzymol. *185*, 60-89.

Tabor, S. (1990) Expression Using the T7 RNA Polymerase/Promoter System. In *Current Protocols in Molecular Biology*, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, eds. (New York: Greene Publishing Associates and Wiley-Interscience), pp. 16.2.1-16.2.11.



©2000-2006 Invitrogen Corporation. All rights reserved.

For research use only. Not intended for any animal or human therapeutic or diagnostic use.